Immobilization of Enzymes onto Modified Polyacrylonitrile Membranes: Application of the Acyl Azide Method

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SYNOPSIS

Chemical reactions toward acyl azide activated polyacrylonitrile (PAN) and conditions for membrane surface modifications are described. Ultrafiltration (UF) membranes were prepared from PAN homopolymer and copolymer with methyl acrylate. Besides hydrazide formation and nitrosation, a new method to introduce acyl azide groups into carboxyl modified PAN, using azido transfer with diphenyl phosphoryl azide, was developed. Chemical conversions were characterized, especially with Fourier transform infrared spectroscopy. The heterogeneous modifications are not chemically selective due to side reactions and/or incomplete conversion. The pore structure is altered predominately via modified polymer swelling causing changed UF fluxes and selectivities. However, for the modification via PAN reaction with hydroxyl amine, acid hydrolysis, and azido transfer, the initial membrane separations performance is qualitatively preserved. Using the acyl azide method, amyloglucosidase (AG) (EC 3.2.1.3) was immobilized onto the modified PAN UF membranes, enabling hydrolysis of starch or maltose to glucose. Enzyme activity was assayed depending on previous chemical modification (azide content) and immobilization (pH) conditions as well as hydrolysis parameters (substrate, conversion during diffusion or UF). The best results (up to 600 mU/cm^2 at 40°C and pH 5.0) were obtained after modification of PAN membranes via carboxyl creation and azido transfer. AG covalently bound to PAN is not influenced much in its catalytic properties (K_m = 3.48 and 3.1 mmol/L for free and bound AG, respectively, with maltose at 40°C and pH 5.0). Under UF conditions, AG effective activity can be improved by the convective flow through the membrane. UF selectivity for the polymer starch determines effective substrate concentrations in the membrane, thus affecting observed activities and product purities in the filtrate. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

The immobilization of enzymes has already found numerous applications. By this means, expensive biocatalysts become reusable, and they may be applied in high local concentrations and in continuous flow systems. However, with enzymes bound to a surface or restricted within a matrix, reduced material transport rates as compared with the dissolved native enzymes often are a problem.[1,2]

In the biotechnology industries, it is necessary to concentrate and isolate valuable products of biocatalytic reactions. Downstream processing with membrane separation operates under very soft conditions, and consequently it is already established in many cases.[3] Enzyme membrane reactors[4] combine biocatalytic conversion and separation.[3,5] Until now, reactions that are equilibrium restricted under conventional conditions dominate in applications of such reactors. Namely, the selective permeation of product through the membrane (usually by diffusion) provides a higher yield. Highly improved transport rates can be realized by convection through appropriate microporous membranes. This

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makes such structures interesting as alternative matrix, for example, in cases of high diffusional resistances with conventional immobilizates[6] or when product inhibition is involved.[7] Also, for example, it has been shown, with intermolecular transglycosylations using cyclodextrin glucanotransferase, that by regulation of both the amount of immobilized enzyme and the residence time in the enzyme membrane, the formation of a desired product, cyclodextrin, can be favored compared with other reactions.[8]

By immobilization of enzymes on synthetic microfiltration or ultrafiltration (MF or UF) membranes, it is possible to integrate both functions (i.e., efficient biocatalysis and separation) within one structure.[3,5] However, there are no general strategies for the design and preparation of such enzyme UF membranes, and their possibilities and eventual limitations are not fully evaluated yet.

Covalent enzyme immobilization onto the membrane pore surface is favored if operation at high fluxes is desired. However, activity decay due to chemical reaction of the enzyme may occur.[1,2] Synthetic polymer membranes for MF or UF usually do not contain any or enough reactive groups; hence, membrane surface modification is necessary to enable covalent binding. This can be done either before[9-12] or after membrane formation.[13,14]

The acyl azide method is known as an efficient and relatively mild way to accomplish covalent protein immobilization onto solid carriers.[1] For almost 50 years,[15] it has been used to bind enzymes to different solid materials such as natural or synthetic polymers or hydrogels and porous glass.[16-19] After various premodifications, depending on the nature of the carrier, one general activation procedure is applied; carboxylic acid ester or amide are converted into hydrazide and these subsequently with nitrosyl ions to acyl azide groups.[15-19]

Diphenyl azido phosphate (DPPA)[20] is known as an efficient reagent for the conversion of carboxyl into acyl azide groups.[21] Its typical applications are solution reactions, including peptide synthesis.[22] To our knowledge, DPPA had not been applied before for creation of acyl azide groups on solid carriers.

We prepare and use UF membranes made from polyacrylonitrile (PAN).[23,24] This is a polymer with rather high chemical resistance. Thus, quite drastic reaction conditions are necessary to create appropriate additional functional groups (for a review cf. reference 25). For covalent binding of enzymes, the latent carboxyl reactivity of nitrile side groups of the polymer should be explored.

The motivation of this work was to combine the advantages of covalent enzyme immobilization with the separation capability and high flux of UF membranes. For that purpose, membranes with different mean pore diameters, and, hence, permeabilities and selectivities, should be activated by chemical surface modifications. Various membrane formation and chemical modification strategies were compared with the objective to apply one coupling method: protein coupling to acyl azide groups on the membrane surface. One enzyme as a model system should be immobilized. In a later stage of the project, we describe the influences of membrane pore structure and surface chemistry, including the coupling method, together with the operation conditions (flux, residence times) onto the efficiency of enzyme UF membranes for simultaneous biocatalysis/separation applications.

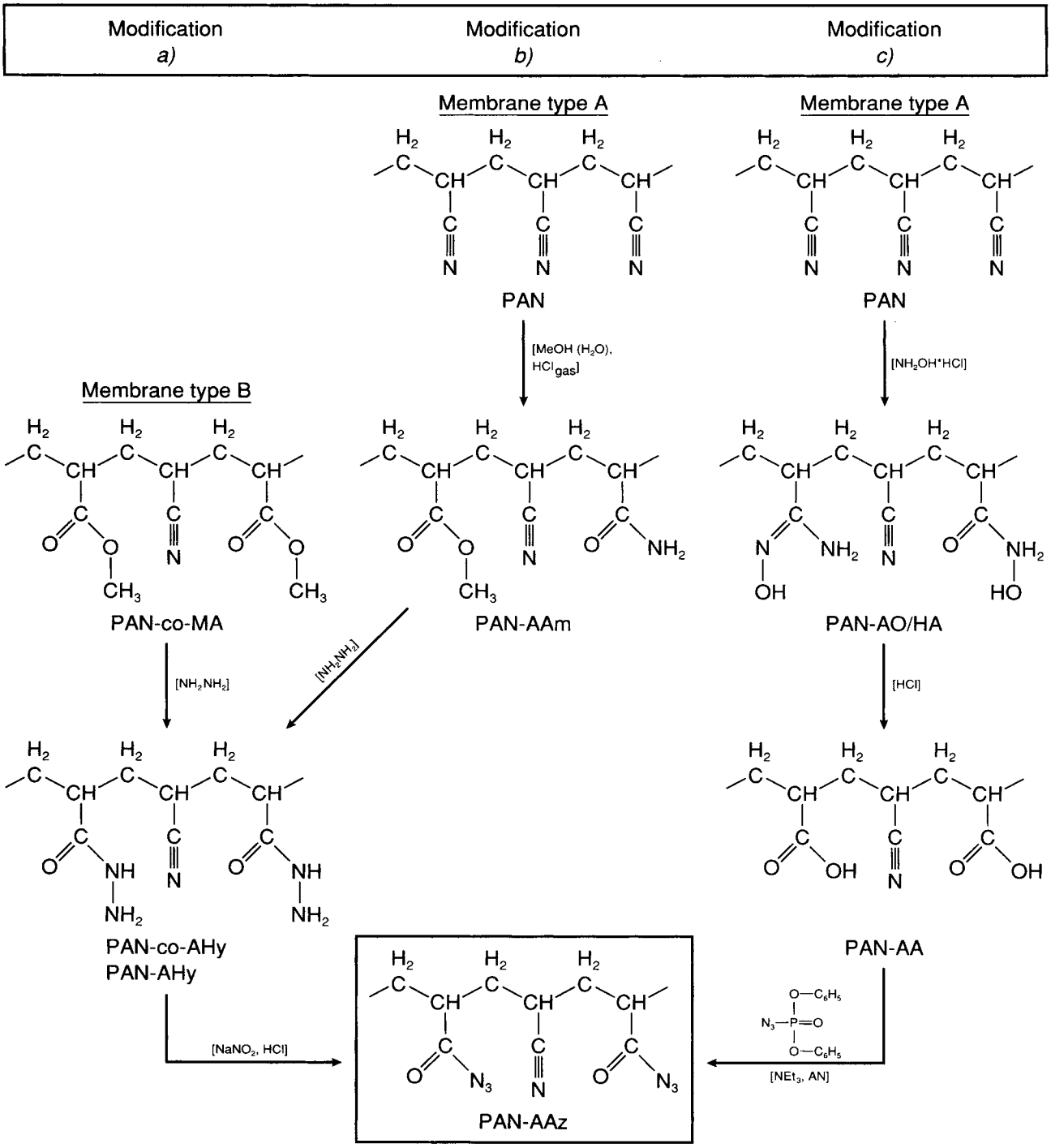
Here we report on different heterogeneous modification procedures for PAN UF membranes. The pathways of the chemical reactions and conditions suited for UF membrane modification are described. Pre- and postformation modification variants were compared. A new method to introduce acyl azide groups into carboxylated carriers using azido transfer with DPPA was developed. Chemical changes were characterized with gravimetry, titrations, and, especially, Fourier transform infrared (FTIR) spectroscopy. The influence of modifications on morphology and permeate flow was studied by electron microscopy and in UF experiments, respectively. Using the acyl azide method, amyloglucosidase (AG) (EC 3.2.1.3) was immobilized onto the modified PAN UF membranes. This model system was used to hydrolyse either the polymer starch or the disaccharide maltose to glucose. Enzyme activity was assayed depending on previous chemical modification (azide content) and immobilization (pH) conditions as well as hydrolysis parameters (substrate, conversion during diffusion or UF).

EXPERIMENTAL

Membranes

Membrane formation

The membranes were prepared from solutions of PAN homopolymer (MW 135,000 g/mol; < 1 wt % vinyl acetate as comonomer; BUNA AG, Schkopau, Germany; type A) or poly-(acrylonitrile-*co*-methyl acrylate) (PAN-*co*-MA; MW 80,000 g/mol; 4.7 wt % ester; type B) with concentrations of 17–



Scheme 1. Pathways of PAN modification.

18 wt % or 8 wt % in dimethyl formamide (DMF) for type A or B, respectively. The solutions were cast (d = 300 μm) at ambient temperature (20°C) and relative humidity (65%) onto the stainless steel belt of a casting machine for continuous membrane production. After a residence time in air of at least 30 s, the casting films were immersed in water as a coagulation bath and held at 18°C. Finally, the membranes were annealed in hot water at 90°C for 10 min. This process yields membranes with maximum relative permeability variations of ±15%.[23]

Membrane modifications

Three different modification procedures were used [Scheme 1, modifications (a–c)]. In modification a, the type B membranes were transformed into activated acyl azide carriers by subsequent treatment with hydrazine hydrate (>99%) for 0.5 h at 40°C and then with a solution of 0.5*M* sodium nitrite and 0.5*M* HCl in water for 0.5 h at 0–5°C (similar to references 16–18). For modification b, type A membranes were modified using methanol saturated with HCl gas (for 3 h at 25°C), a procedure aiming at the formation of methyl ester but yielding amide structures. Acyl azide groups were obtained by reaction with hydrazine hydrate (>99%) for 3 h at 60°C and then again with nitrosyl (0.5*M* sodium nitrite and 0.5*M* HCl in water; 0.5 h, 0–5°C, similar to references 16–18). Alternatively, in modification c, type A membranes were treated with a hydroxylamine hydrochloride solution (100 g/L; pH 6) at 62.5°C for 30 min[26–29] and then carefully washed with water. For further hydrolysis, these membranes were immersed in 2*M* HCl at 80°C for 5 h; the resulting "carboxyl modified" samples were leached in water.[29] Acyl azide was formed as follows.[29] Single membranes were put into a filter holder (SM 16308 [*d* = 100 mm; solution reservoir of 400 mL] or SM 16278 [*d* = 47 mm]; Sartorius GmbH, Göttingen, Germany) and rinsed two times with acetonitrile (AN). Thereafter, a 160 m*M* triethylamine solution in AN was passed through the membrane. DPPA (160 m*M*; Merck, Darmstadt, Germany) was added to this solution, which then again was forced through the membrane. This filtration step was repeated several times; in between the membrane was immersed in the DPPA–Et_3N solution until 48 h had been accumulated. The temperature was kept at 37°C throughout the entire reaction. Finally, the membrane was rinsed four times with AN and two times with diethyl ether and then dried.

Analyses

Gravimetric determinations were done with a microbalance (MP 20; Feinmechanik, Freiberg, Germany). Elemental analyses were performed using an automat 240 (Perkin-Elmer Co., Überlingen, Germany). UV-vis spectra were recorded using a model PU 8735 (Pye Unicam, Cambridge, UK) or a UVIKON 930 (Kontron Instruments, Milano, Italy).

Carboxylic acid titration

Membrane samples of 30–100 mg were dissolved in 5.0 mL dry DMF for 48 h. Using a microburette, the samples were then slowly titrated with 5 or 10 m*M* NaOH solution (indicator bromthymol blue). The blank value of DMF (usually < 0.2 m*M*) was subtracted. The titration of poly(acrylic acid) (Aldrich) under the same conditions gave (10.7 ± 0.8) mmol/g, what corresponds to 77% of the theoretical carboxylic acid content. This was used to correct membrane data.

FTIR measurements

All IR spectra were obtained by the attenuated total reflection (ATR) technique using a Nicolet Magna 550C FTIR spectrometer with MCT/A detector and 4 cm^{-1} resolution and a horizontal ATR device with KRS-5 crystal (Spectra-Tech, Solingen, Germany).

Scanning electron microscopy (SEM)

The wet membrane samples were dried by solvent exchange with ethanol and finally with diethyl ether. Specimen were prepared by fracturing at liquid nitrogen temperature and coating with gold. Membrane cross sections were analyzed using an SEM BS 340 (Tesla, Czech Republic).

Azide content determination

Dry acyl azide activated UF membranes (PAN-AAz) can be stored at 4°C for about 1 week. The azide content was determined by a photometric method.[29,30] About 15 mg of dry membranes were hydrolyzed in 1 mL 0.1*M* NaOH at room temperature for 48 h and then diluted with 1 mL water. Aliquotes (0.1 mL) of this solution were added to 4.9 mL of 0.1*M* aqueous $NH_4Fe(SO_4)_2$ solution. After 2 min, the UV-vis absorption was measured at 458 nm. The azide content calculation was based on a calibration curve obtained with sodium azide solutions. Values from at least three samples were averaged, giving an accuracy of ±15%.

Preparation of poly(acrylic acid-co-acryloyl azide) by heterogeneous azido transfer reaction

Poly(acrylic acid) (28.8 mg; MW ca. 200,000 g/mol, Aldrich Co.) was suspended in 2 mL AN containing 0.8 mmol triethyl amine. Then, a solution of DPPA (1.59 mmol) in 2 mL AN was added and the mixture was shaken for 6 days at 37°C. Thereafter, the solid was filtered off, washed four times with 5 mL AN and two times with 5 mL diethyl ether, and dried *in vacuo* at room temperature [IR(KBr): 2140 cm^{-1}; azide content: 4.37 mmol/g].

Heterogeneous or homogeneous conversion as function of solution pH, temperature, UV irradia-

tion, or presence of *n*-butyl amine was followed with IR spectroscopy.

Enzyme membranes

Enzyme

AG from *Aspergillus niger* (1.4-α-D-glucan glucohydrolase; EC 3.2.1.3.) with a specific activity of 75 U/mg (at pH 4.8 and 60°C for starch) was purchased from Merck. The enzyme has a MW of ca. 75,000 g/mol containing 10 wt % carbohydrate.[31]

Enzyme immobilization

The enzyme solution (10 or 50 mL, respectively; c_o = 10 g/L in 67 m*M* phosphate buffer, pH 8.0 or in some cases 6.5 or 5.0; 20°C) was forced at low pressure ($p < 0.1$ MPa) through the membranes (effective area 15.6 or 37.4 cm^2, respectively) in inverse orientation fixed in a UF cell (that means, the membrane sublayer was exposed to the solution). Depending on the membrane permeability, this step lasted 4–5 h. Thereafter, with the membrane in the normal orientation (skin layer toward the solution), an intensive rinsing was carried out with buffer (first pH 8.0, then pH 5.0) until no activity could be measured in the filtrates. Enzyme loading of the membranes was estimated from the mass balance, based on UV spectroscopy of protein in all solutions (AG absorption coefficients $\varepsilon_{280\,nm}$ = 1.233 L/g · cm at pH 5.0 and $\varepsilon_{280\,nm}$ = 1.164 L/g · cm at pH 8.0).

Activity assays

The polymer starch (soluble acc. to Zulkowsky, MW 22,000 g/mol measured by GPC) and the disaccharide maltose were used as substrates (both purchased from Merck). Substrate solutions were prepared in phosphate buffer (70 mM, pH 5.0) in concentrations of 20 g/L for standard activity assays or between 1 and 40 g/L for enzyme kinetic studies. All reactions were performed at 40°C. Glucose product concentrations were determined using an electrochemical glucose analyzer (Prüfgerätewerk Medingen GmbH, Freital, Germany) or the photometric methods according to Sumner[32] (for starch) or Bergmeyer[33] (for maltose).

Evaluation of UF membrane performance

For characterization of the UF membranes, a stirred UF cell (effective membrane area 37.4 cm^2; Berghof, Germany) was used with a pressure of 0.3 MPa and at room temperature. Usually, dextrane (MW 70,000 g/mol; Pharmacia) was the test substance used as 1 g/L solution (c_o) in water. Sugar concentrations were analyzed with a polarimeter (model 241, Perkin Elmer Corp.).

All selectivities φ were calculated according to

$$\varphi = (c_{ret} - c_{filtr})/c_{ret} * 100\%$$

with c_{filtr} dextrane concentration in the filtrate fractions and c_{ret} dextrane concentration in the retentate (either determined directly or calculated from c_o and c_{filtr} using the mass balance).

Separation curves

The determination of the pore size distribution was done by UF of aqueous poly(ethylene glycol) mixtures having a broad molecular weight distribution and subsequent analysis of feed and filtrate samples with gel permeation chromatography.[34] Retention values depending on M_{PEG} were computed from filtrate and feed chromatograms. Based on this data and the relationship between test substance molecular weights and diameters (d_{PEG}[nm] = 0.1474* $M_{PEG}^{0.3915}$), the separation curves or the pore size distributions were calculated.[34]

Catalysis UF experiments

Catalysis UF experiments were performed using a stirred micro-cell (effective membrane area 15.6 cm^2) kept at 40°C with an external thermostat. Fifty milliliters of starch solution (20 g/L) was filtered at a rate of 0.6 mL/min, maintained by adjusting the pressure, until 30 mL filtrate were accumulated. Glucose was assayed with the analyzer as described above. Starch was determined as glucose after total hydrolysis using 2 mg AG/200 μL solutions (pH 5; 5 h at 40°C) based on a starch concentration calibration curve.

The calculations of starch retention and conversion, based on glucose and starch analyses in feed, filtrate, and retentate, were done using the following assumptions. First, active enzyme is located only in the sublayer of the membrane (i.e., not on top of the active layer). Second, there is back diffusion (through the active layer) into the feed reservoir only for glucose but not for starch. Finally, as a consequence, glucose found in the retentate originates from permeable starch and is accordingly considered for determination of retention and conversion.

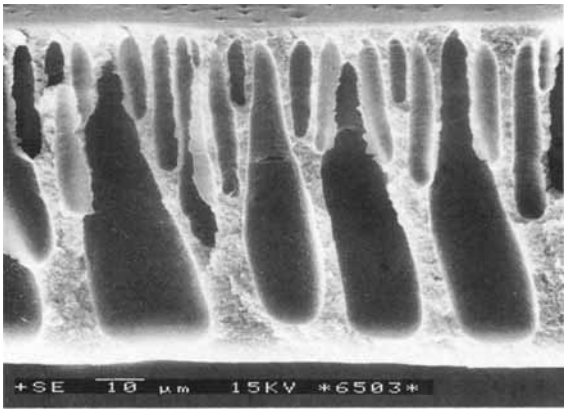


Figure 1 SEM micrograph of the cross-section of PAN UF membrane type A.

RESULTS AND DISCUSSION

Membranes

Membranes were prepared by the wet-phase inversion process from PAN and from PAN-*co*-MA, using DMF as solvent and water as coagulant. Typical SEM micrographs of membrane cross sections are shown in Figures 1 and 2. The macropores of the PAN membranes (type A; made from about 17.5 wt % solutions) have a finger-like shape. The pore size gradient from the sublayer surface toward the active layer ("asymmetric structure"; Figures 1 and 2) is characteristic for many UF membranes. Because of the limited solubility of PAN-*co*-MA in DMF, it was necessary to use low polymer concentrations such as 8 wt %. A much more open sublayer structure is the consequence (type B; Fig. 2). However, PAN-*co*-MA membranes (type B) may exhibit a more pronounced "fast precipitation" structure with a thin relatively dense skin layer.[35] This is due to worse solubility and irrespective lower concentrations compared with PAN membranes (type A) under otherwise similar formation conditions (solvent and coagulant). Indeed, water permeabilities (135–610 L/m^2 h at p = 0.3 MPa) and selectivities (18–55% for dextrane with MW ~ 70,000 g/mol; Table I) indicate that all studied UF membranes* are in the same order of magnitude with regards to active layer average pore size/porosity.

Hence, UF membranes with quite similar (not equal) pore structure but different chemical composition were available for the evaluation of the three different modification strategies (Scheme 1).

* Three lots of type A membranes from different preparations (slightly varied PAN concentration) were used throughout the studies (cf. Table I; Fig. 9).

Heterogeneous membrane modifications

Modification a

With PAN-*co*-MA membranes (type B), containing methyl ester groups, it was expected that the established activation procedure for the acyl azide method, hydrazide formation and nitrosation [cf. Scheme 1(a)], can be applied easily. Structure conversions of PAN-*co*-MA membranes could be identified in the ATR-IR spectra; acyl azide structures were indeed formed in a two-step reaction (Fig. 3). However, the membranes lost much of their mechanical stability. This was indicated by an increased brittleness, and hence the samples were prone to the formation of macroscopic defects. Also, the water fluxes of the acyl hydrazide membranes (PAN-*co*-AHy) were strongly reduced as compared with the parent samples (cf. Table I).

Modification b

The attempted alcoholysis of PAN membranes [type A; cf. Scheme 1(b)] was obviously not a well-defined reaction, as indicated by the ATR-IR spectra (Fig. 4). The formation of ester and predominately amide moieties (PAN-AAm) was indicated by typical carbonyl stretching bands around 1735 and 1665 cm^{-1}.[36] On the other hand, the cyclization of adjacent nitrile groups (typical reaction of PAN) seemed to be involved as well (broad IR absorption increases between 1595 and 1655 cm^{-1} [37]). The further transformation, especially of amide groups, required drastic conditions, damaging the porous structure of the membranes. This resulted in a poor mechanical stability of the modified samples. In addition, the nitrile groups were also involved in the reaction with hydrazine hydrate indicated by the strong reduction

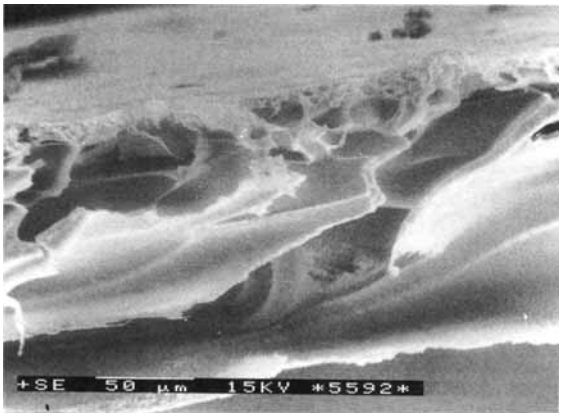


Figure 2 SEM micrograph of the cross-section of PAN-*co*-MA UF membrane type B.

of the ν(CN) absorption intensity, and, most important, the acyl azide absorption intensity in the activated samples (PAN-AAz) was rather low (Fig. 4). In summary, the overall modification procedure has a limited efficiency, step-wise PAN cyclization, and degradation are competing processes.

Modification c

PAN UF membranes with carboxylic acid groups (PAN-AA). Another approach, starting with PAN (type A), was to use the well-known reaction of nitrile with hydroxyl amine[28] [cf. Scheme 1(c)], which had been used, for example, to make metal affinity fibers[38] or nanofiltration membranes.[39] Based on the ATR-IR spectra (Fig. 5), structures formed from nitrile were interpreted as mixture of amide oxime and hydroxamic acid (PAN-AO/HA; ν(NO) at 910 and 930 cm^{-1} along with a broad carbonylic band centered at 1660 cm^{-1} [26]). Typical quantitative results for PAN membrane conversion are shown in Table II. Up to 20% of the nitrile groups could be converted without destruction of the membrane's integrity (Table II). With PAN fibers under the same reaction conditions, including conversion time, Weiping et al.[38] observed much lower conversions, what may be due to the high specific surface area of the PAN UF membranes. SEM analyses did not show any change in morphology (at the given resolution) compared with the parent samples. However, the cut-off is changed from 168,000 g/mol for the parent to 1600

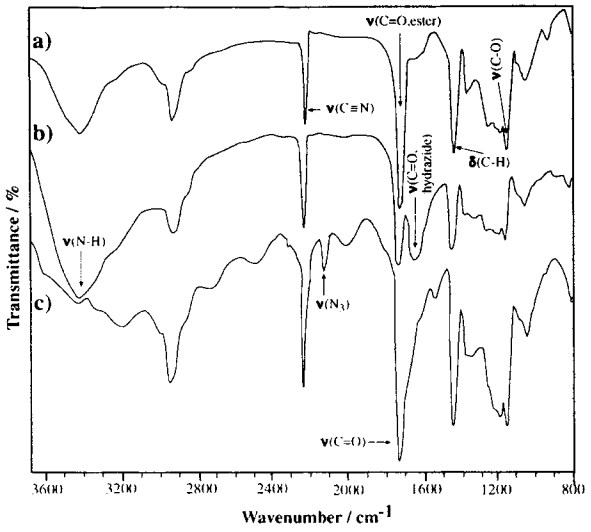


Figure 3 ATR-IR spectra (KRS 5, 45°) of products obtained during Modification a of PAN-*co*-MA UF membranes (type B), designated as (a) PAN-*co*-MA, (b) PAN-*co*-AHy, (c) PAN-AAz (cf. Scheme 1).

Table I Summary of UF Data for Original and Modified PAN Membranes (Types A and B)

Membranes	Water Flux J_W (L/hm^2)		Filtrate Flux J_V (L/hm^2)	Selectivity φ_{M70} (%)
	type A, 1	*type A, 2*	*type A, 1*	*type A, 1*
PAN	135	340	75	25
PAN-AO/HA	35	17	21	75
PAN-AA	67	158	44	51
PAN-AAz	34	n.d.	38	88
PAN-AG = Modification c	14	30-45	15	95
	type A, 3			
PAN	202		63	54
PAN-AAm	266		99	50
PAN-AHy	11		n.d.	n.d.
PAN-AG = Modification b	18		15	61
	type B			
PAN-*co*-MA	611		64	23
PAN-*co*-AHy	108		n.d.	n.d.
PAN-*co*-AG = Modification a	78		40	11

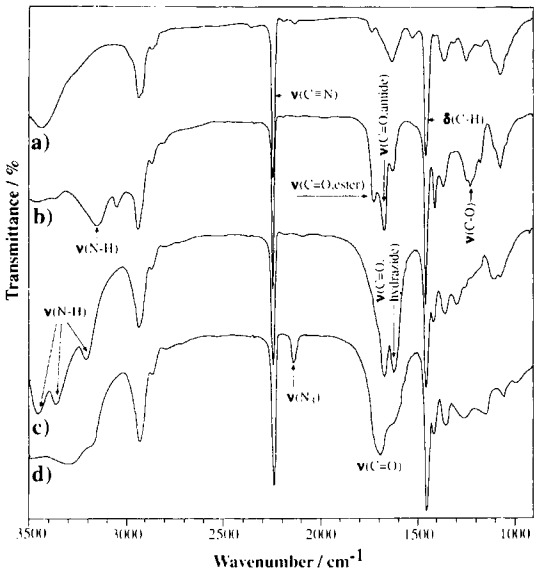


Figure 4 ATR-IR spectra (KRS 5, 45°) of products obtained during Modification b of PAN UF membranes type A, designated as (a) PAN, (b) PAN-AAm, (c) PAN-AHy, (d) PAN-AAz (cf. Scheme 1).

g/mol for the modified membrane (Fig. 6). Separation curves show a shift, indicating that the average pore diameter in the active layer is reduced from 10.0 ± 0.7 to 1.2 ± 0.3 nm. The reduced slope for PAN-AO/HA membranes as compared with PAN membranes is explained with a broader active layer pore size distribution. These results show that the modified active layer pore structure is largely governed by swelling of the hydrophilic polymer surface (AO/HA) covering the solid bulk polymer.

Further hydrolysis produced carboxylic acid groups identified with ATR-IR spectroscopy (ν(C—O) at 1210 cm^{-1} and ν(C=O) at 1690 cm^{-1}; Fig. 5). With increasing intensity of the PAN-AA bands, the absorptions of PAN-AO/HA decrease. In SEMs, the macroporous PAN structure (cf. Fig. 1) seemed to be preserved in PAN-AA membranes. Water flux was improved again as compared with PAN-AO/HA (cf. Table I). Also, it can be seen that the carboxylic acid amount is a function of the previously produced AO/HA content, which itself is dependent on the reaction time with hydroxyl amine (Table II). Stronger reaction conditions improve the yields, and about 25% of the amide oxime/hydroxamic acid can be hydrolyzed. Thus, carboxyl amounts of up to 0.8 mmol/g (=283 μmol/cm^2) were obtained.†

† Using membranes with a higher initial permeability (type A, 2; cf. Table I), higher degrees of conversion under the same conditions were observed (cf. Table II); up to 12.2 mol % (2.0 mmol/g; 710 μmol/cm^2) carboxyl groups (—AA) were obtained.

Heterogeneous carboxylic acid-carbonyl azide conversion using DPPA. For application of the acyl azide method (via modification c), conversion of carboxylic acid to carbonyl azide was required. We selected the azido transfer reagent DPPA, which previously had been used in solution reactions exclusively.

It was necessary to use a solvent for membrane modification that does not swell or damage the membrane pore structure and that accelerates the reaction by solvent polarity.[21] Moreover, it should not react to acyl azide solvolysis products. AN was found to fulfill all requirements satisfactorily. The possibility to achieve azido transfer onto surface aliphatic carboxyl groups was verified with poly(acrylic acid) of high Mw; about 35 mol % azide yield were obtained in the heterogeneous reaction at 37°C within 6 days.

Typical quantitative results for the influence of reaction conditions during preparation of acyl azide activated PAN membranes are shown in Table III. Starting with the same PAN-AA membranes (4.9 mol % COOH, from type A, 1), the excess of DPPA and triethylamine, the total reaction time, and repeated flushing of the reactants (accumulated time of convection; cf. Table III) through the membranes can be used to improve the yield to 60–70% azide relative to the carboxyl content on the membrane surfaces. Acyl azide groups on the membrane surface are easily identified in the ATR-IR spectra (Fig. 5). A correlation between azide content determined via hydrolysis and photometry[30] and the apparent con-

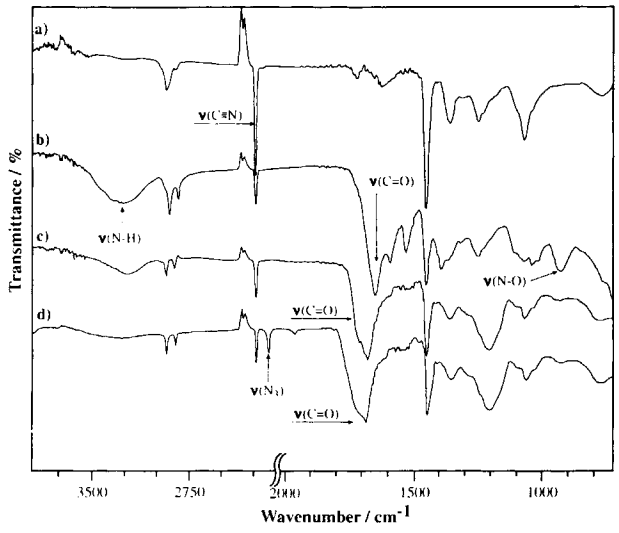


Figure 5 ATR-IR spectra (KRS 5, 45°) of products obtained during Modification c of PAN UF membranes type A, designated as (a) PAN, (b) PAN-AO/HA, (c) PAN-AA, (d) PAN-AAz (cf. Scheme 1).

version to acyl azide from ATR is observed (Table III). From PAN UF membranes type A, azide amounts between 0.5 and 10 mol % (0.08–1.6 mmol/g; 28–566 μmol/cm^2) could be prepared and used in further immobilization experiments (cf. Fig. 7).

Solid polymeric acyl azides, either from PAA or from PAN-AA membranes, could be stored in dry state for at least 5 days at 10°C without detectable decomposition. Hydrolysis in water (pH 6) was slow (10% conversion in 24 h), whereas in phosphate buffer (pH 8) it was much faster (40% conversion in 0.5 h). Thermal or photochemical decomposition yielding isocyanates supported the structure assignment. Amides were formed with *n*-butyl amines in a fast clean reaction. The remarkably high stability of the synthesized polymeric aliphatic acyl azides can be compared with similar data found for dissolved higher MW aliphatic acyl azides (oligopeptides)[40] or aliphatic acyl azide groups on a solid support.[41] In contrast, attempts to synthesize poly(acrylic acid azides) from the hydrazide precursors yielded unstable products that already started to decompose during the preparation.[42] In conclusion, heterogeneous activation of carboxylated membranes with DPPA, yielding thermally quite stable acyl azides is possible. However, solvent exchange from water to a polar nonnucleophilic solvent for the reactants and a nonsolvent for the membrane was necessary, and reaction times were quite long.

In conclusion, all three different approaches provide the possibility to introduce reactive acyl azide groups into the surface of modified PAN UF membranes (cf. Scheme 1). However, the reaction sequences must be carefully evaluated with respect to reaction conditions and degree of conversion because the creation of reactive surface groups has also influence onto the bulk material stability. In particular, further conversion of PAN hydrolysis products (from type A; part of modification b) due to the low reactivity and the consequently harsh conditions is not an appropriate choice for the modification of UF membranes, because about 95% of the original permeability are lost during this step. In the other cases, damage of the membranes can be limited. Due to better suited functional groups (ester instead of less reactive amide), modification a, starting with copolymer membranes type B, is relatively well defined. Also, modification c, starting with PAN type A, gives good to moderate yields in every step; the specificity of the azido transfer provides high yields of reactive acyl azide. In all cases, swelling of modified surfaces (which are more hydrophilic than PAN) in aqueous medium may reduce the reaction rate and by this means the degree of conversion in further modification steps. Changes in membrane permeability and selectivity are thought to be caused by active layer swelling effects predominately, but there is a loss of mechanical stability to a certain degree as well.

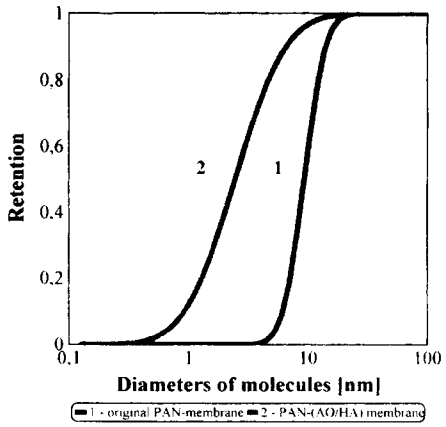


Figure 6 Separation curves (from UF of PEG mixtures) for original PAN and PAN-AO/HA membranes (type A; modified via c; cf. Scheme 1).

Enzyme immobilization

Acyl azide membranes from all three modification sequences were used to prepare AG membranes. For

Table II Quantitative Results for Modification c of PAN UF Membranes (Type A, 1)

Reaction With Hydroxyl Amine: Time at 62.5°, *t* (min)	PAN Nitrile Conversion (mol %)[1]	Acid Hydrolysis: 2*N* HCl; Reaction Conditions	PAN-AA Carboxylic Acid (mol %)[2]
10	7.3	2 h; 65°C	0.6
30	19.9	2 h; 65°C	1.4
30	19.9	5 h; 85°C	4.9

Reaction with hydroxyl amine (PAN-AO/HA) and subsequent acid hydrolysis yielding PAN-AA membranes (cf. Scheme 1).
[1] From gravimetry and ATR-IR spectra, AO/HA content verified with elemental analysis.
[2] From carboxylic acid titration, verified with gravimetry.

Table III Quantitative Results for Modification c of PAN UF Membranes (Type A, 1)

DPPA : COOH Ratio ($c : c$)[1]	Reaction Time (at 37°C) With Transport in/through Membrane By		IR Absorbance Ratio ($N_3 : CN$) From ATR[2]	Acyl Azide Content (mol %)
	Diffusion, t (h)	Convection, t (h)		
11 : 1	16	8	0.103	0.5
11 : 1	40	8	0.205	1.0
37 : 1	48	0	0.266	1.6
37 : 1	46	2	0.605	2.9

Preparation of acyl azide activated PAN membranes (PAN-AAz) from PAN-AA membranes via azido transfer with DPPA (cf. Scheme 1).

[1] Based on carboxyl content in PAN-AA membrane (cf. Table II); molar ratio DPPA : Et_3N 1 : 1; both 160 mM in AN.

[2] cf. Figure 5.

that purpose, protein solution was ultrafiltered through the activated membranes in reverse orientation (sublayer exposed to enzyme; cf. Figures 1 and 2). By this means, the deep filtration effect was used to enhance the binding probability to the intraporous membrane surface[‡] (cf. references 6, 9–11). After intensive rinsing, AG membranes with stable activity were obtained from all activated precursor types. Within one activity assay experiment, the conversion stopped after the immobilizates were removed from the substrate solutions; hence, no enzyme release from the membranes occurred during the assay. Repeated assays within up to 4 weeks yielded only minor drops (<10%) in activity. Without acyl azide activation of the membranes, no stable AG membrane activity could be observed. Thus, the covalent character of the immobilization is demonstrated indirectly.

Overall, AG membrane activities under standard conditions (starch; c_o = 20 g/L; pH 5.0; 40°C) depending on membrane modification before activation and on immobilization conditions are summarized in Table IV. For PAN-AAz membranes, prepared with DPPA via c, the influence of azide content and pH during immobilization onto activity is plotted in Figure 7. It can be seen that raising the pH from 5.0 to 8.0 strongly increased AG activity of the immobilizates (Fig. 7). This is explained by the improved protein amino group reactivity with acyl azide to form amide bonds with the membrane surface. However, further pH increase will not be advantageous, because of drastically enhanced acyl azide hydrolysis rates (cf. above).

A comparison of the three different membrane modification approaches clearly shows that modification c enables by far the highest AG activities, followed by a and b (cf. Table IV). Reasons can be seen in two factors. First, different activities could be due to differences in azide content. Second, variations of the specific surface accessible for the protein may be involved as well. Membranes modified via b clearly had the lowest permeability compared either with the other precursor or enzyme membranes (cf. Table I). The results for modification b can be explained by accessibility restrictions toward protein binding along with the poor chemical specificity, yielding small amounts of coupling groups (cf. above). Comparing routes a and c, higher permeabilities, and consequently better accessibility for AG, are seen for a (cf. Table I). An influence of substrate transport limitation onto activity (expected to be larger for the lower flux PAN-AAz prepared via c; cf. Table I) does not explain the observed differences. This leads to the conclusion, that membranes prepared via a may contain less coupling groups. This may be due to the low ester content of the bulk polymer (4.7 wt %; = 3.0 mol %), which is obviously not entirely exposed to the surface and also not completely converted to acyl azide. Note that via modification c, up to 10 mol % acyl azide were obtained (Table IV, Fig. 7). In addition, the results agree with both the common observation that activation via hydrazide/nitrosation (Modification a) yields "unstable" carriers[15–19,42] and our finding that acyl azide formation in nonnucleophilic organic solvent (Modification c) with subsequent drying yields quite stable activated supports for covalent coupling.

[‡] For PAN-AAz membranes in normal orientation, the UF retention of AG is quite high (ϕ_{AG} = 50–70%); consequently, the loading of the entire membrane structure with enzyme is not effective.

Regarding the influence of acyl azide content onto AG activity obtained under the same immobilization conditions, there is an optimum, in our case observed around 7 mol % acyl azide (cf. Fig. 7). The drop with higher acyl azide amounts may be explained with double or multiple binding of protein enforced by the higher surface concentration of reactive groups. This may cause changes of the active conformation or reduced flexibility, hindering the formation of the enzyme substrate complex and/or the release of the product.[2]

At this stage of the project, protein loadings could only be estimated from the protein mass balance; typical values for the AG membranes are about 0.05 $\mu g/cm^2$ (type B) and 0.2–0.5 $\mu g/cm^2$ (type A). However, this indirect measure is not accurate enough; therefore, we are currently developing an assay for quantitative determination of covalently bound enzyme on modified PAN carriers based on acid protein hydrolysis and subsequent high-performance liquid chromatography analysis (cf. references 43 and 44).

To get more information on the state of membrane immobilized AG, kinetic parameters were determined by variation of substrate concentration and assuming Michaelis–Menten kinetics.[2] We selected membranes with comparatively high (apparent) activities under "standard" conditions and the low molecular weight substrate maltose to eliminate the influence of hindered substrate diffusion into the porous carrier as much as possible.[45] The results, including data for the native enzyme in solution, are shown in Figure 8. K_m is almost unchanged (3.48 mmol/L for dissolved, 3.13 mmol/L for bound AG), indicating that the affinity of the covalently bound

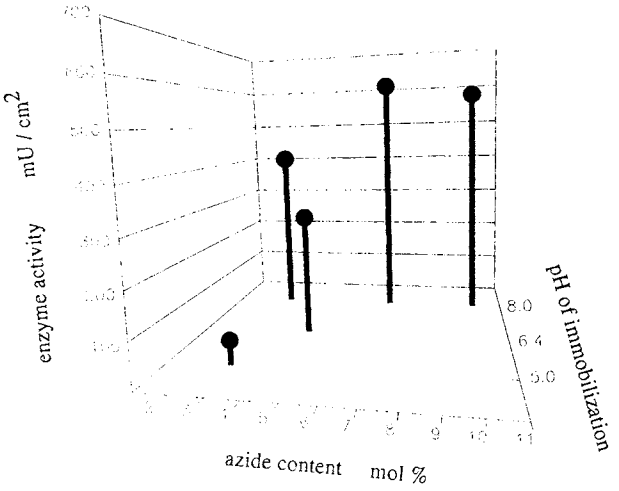


Figure 7 Activity of AG membranes prepared from PAN-AAz UF membranes (type A; modified via c) depending on acyl azide content and pH during immobilization.

AG for the substrate is basically preserved. This is in agreement with previous observations[19] that irrespective of its covalent character, coupling via acyl azide is a mild immobilization method for AG. Due to substrate size and membrane porosity, along with the estimated high active AG loadings, transport limitation does not significantly affect the observed activity.

UF performance of enzyme membranes

Finally, the UF data of the enzyme membranes were compared. The specific impact of the different mod-

Table IV Activities of AG Membranes Prepared From UF Membranes Type A and B and Using Different Modification Strategies

Modification and Initial Membrane	Acyl Azide Content (mol %)	pH During AG Immobilization	Apparent AG Activity (mU/cm^2)
Modification a			
PAN-*co*-MA; type B	n.d	8.0	45
Modification b			
PAN; type A, 3	n.d.	8.0	36
Modification c			
PAN; type A, 1 and 2	0.0	8.0	0
	0.5	5.0	13
	2.9	5.0	50
	2.9	8.0	405
	4.5	6.4	280
	6.9	8.0	600
	10.0	8.0	570

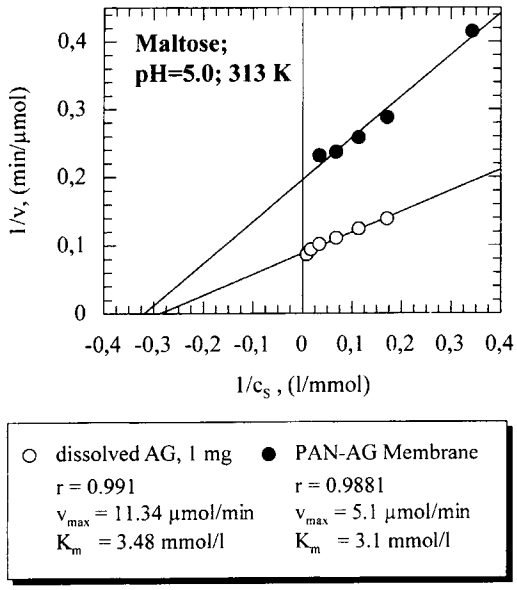


Figure 8 Lineweaver–Burk plots for dissolved and on PAN-AAz membrane (type A; modified via c) immobilized AG.

ification strategies (a–c) onto permeability had been discussed above. Nevertheless, the permeability of the unmodified membranes determines the UF behavior of the AG membranes as it is demonstrated for all studied membranes (Fig. 9; cf. Table I). From the copolymer (type B), AG membranes with the highest permeabilities were obtained. Note, however, that these membranes have low activity (cf. Table IV). In addition, high UF flux is correlated with low dextrane retention and low flux with high UF selectivity (cf. Table I). Hence, important features of the initial membrane pore structures are preserved throughout the entire procedures for all three modifications.

More evidence for this statement is provided by the comparison of separation curves for an unmodified precursor and the resulting AG membrane (Fig. 10). It can be seen that the AG membrane curve is only shifted toward a lower cut-off, but the slope is unchanged. This means that modification and immobilization of protein evenly reduce the average pore size but do not block active layer pores. It is interesting to note that two membranes (from PAN type A, 2; modified via c), which had been only different in azide content (6.9 and 10.0 mol %), gave AG membranes with similar activity (cf. Fig. 7) but different flux (cf. Tables I and V). The observed lower flux for the higher azide content membrane may indicate more immobilized protein, thus reducing average pore size. However, the activity is

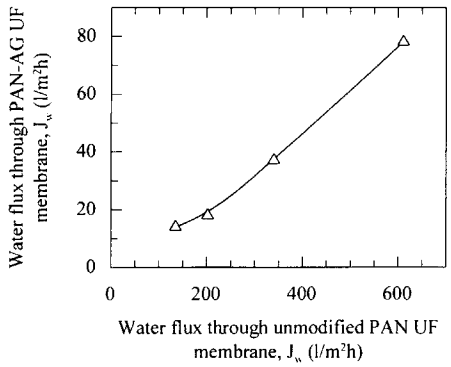


Figure 9 Permeability of AG UF membranes (prepared via a–c; cf. Scheme 1) depending on permeability of the corresponding original membrane (cf. Table I). Pressure 0.3 MPa.

even smaller due to the partial deactivation of bound protein, as invoked above.

Results of catalysis UF experiments with the polymeric substrate starch, using two different AG PAN membranes with high activity under standard conditions, are summarized in Table V. When using the membranes in normal orientation, a quite high starch retention was observed. With the same membranes in reverse orientation (open sublayer facing the feed and active layer at the bottom), the retention is drastically lower. This can be understood if one considers that retention is governed by the active layer pore structure. Starch during permeation through the sublayer is catalytically hydrolyzed, by this means reducing its molecular weight and increasing the probability to pass the active layer. In addition, concentration polarization, induced by the deep-filtration effect, would also reduce retention, but we assume that this cannot account for the large changes.

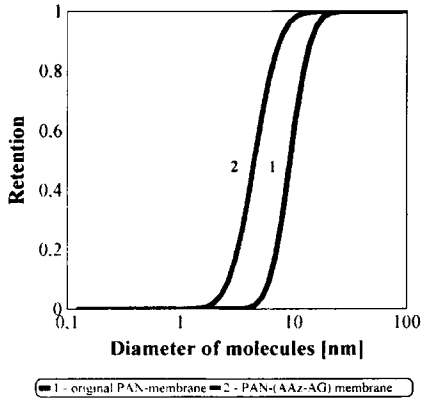


Figure 10 Separation curves (from UF of PEG mixtures) for PAN and PAN-AG UF membrane (type A; prepared via c; cf. Scheme 1).

Table V Results of UF/Starch Hydrolysis Experiments with PAN-AG UF Membranes

	Water Flux J_W at 0.3 MPa (L/hm^2)		Selectivity φ_{starch} (%)		Starch Conversion (%)		Effective AG Activity – UF[2] (mU/cm^2)		Apparent AG Activity – Diff.[3] (mU/cm^2)	
Membrane[1]	–AG,1	–AG,2	–AG,1	–AG,2	–AG,1	–AG,2	–AG,1	–AG,2	–AG,1	–AG,2
Normal orientation	44	30	60	90	36	51	917 (1.53)	810 (1.42)	600	570
Reverse orientation	43	38	14	4	16	35	1065 (1.78)	941 (1.65)		

[1] Membrane PAN-AG,1: from PAN-AAz with 6.9 mol % acyl azide; membrane PAN-AG,2: from PAN-AAz with 10.0 mol % acyl azide (cf. Table IV).

[2] Ultrafiltration: transmembrane flux J_v = 0.6 mL/min = 23.1 L/m^{2}h. Relative improvement in parentheses: [effective AG activity under UF conditions]/[apparent AG activity under diffusion conditions].

[3] Diffusion: "standard" activity assay, cf. Table IV, Figure 7.

However, and most important, in each case the effective AG activity under UF conditions (effective activity) is higher than with the same membrane immersed in substrate solutions where transport occurs by diffusion only (apparent activity; cf. Table V). Starch conversions are lower with the membrane in reverse orientation as compared with the normal one. On the other hand, the effective activities show the opposite trends. These phenomena are explained as follows. With the membranes in reverse orientation, a much higher substrate concentration exists in the membranes compared with the normal orientation (e.g., starch retention of 90% means that the effective substrate concentration in the enzyme membrane is only 10% relative to the feed concentration). This causes increased reaction rates yielding higher effective activities (cf. Fig. 8). For the same reason (higher substrate concentration), the degree of conversion** can drop when the enzyme concentration is not high enough and/or its accessibility by convection is not optimal. Hence, the response of the enzyme UF membrane system using a substrate that is fractionated and hydrolyzed (Table V) is much more complex than, for example, the case of low MW substrate conversion in the presence of an inert substance that is ultrafiltered at the same time.[11]

Comparing the two different AG membranes, it is interesting that the membrane with slightly lower activity and smaller active layer average pore size (PAN-AG,2) gave higher conversion (i.e., product purity) at the same filtration rate. On the other hand, the activity improvement by convection is higher with the more active and larger pore size membrane (PAN-AG,1; cf. Table V). This impact of enzyme UF membrane structure onto efficiency is obviously caused by the interplay of the parameters pore size, enzyme loading and activity, and transport rates, as it is quantitatively analyzed, for example, in the work of Staude et al.[9–11] Further experiments with systematic variation of these parameters will hopefully enable us to optimize enzyme UF membrane performance.

Conclusions

With the polyacrylonitrile surface modification approaches outlined in Scheme 1, it is possible to produce UF membranes with covalently bound enzymes.

In the membrane formation process, the pore structures of active layer, governing the separations performance, and sublayer, macropores later serving as enzyme reactor, can be adjusted. All heterogeneous PAN modifications suffer from only partial chemical selectivity due to side reactions and/or incomplete conversion, and they change the pore structure, predominately via changed polymer swelling because of limited surface selectivity. This always has consequences for UF flux and selectivity. However, conditions can be found where polymer bulk reactions are suppressed and the initial membrane separations performance is qualitatively preserved. An alternative modification approach to create reactive coupling groups is the very surface selective photoinduced graft polymerization.[46]

** The observed starch conversions are based on the content of glucose, final product of the starch hydrolysis cascade, among total sugar in the filtrates and are consequently a measure of product purity.

Acyl azide activated PAN surfaces can be prepared using the conventional acyl hydrazide nitrosation and an alternative approach, namely azido transfer with diphenyl phosphoryl azide onto carboxyl groups. With the latter method, thermally quite stable acyl azides can be obtained and characterized.

AG can be bound to acyl azide activated PAN membranes, yielding variable enzyme loadings and activities. The best results were obtained using modification c (carboxyl creation and azido transfer). This surface modification enables also other coupling reactions (e.g., using carbodiimid chemistry; cf. reference 1). Modifications a and b are much less suited. There are limitations for the *co*-polymer content in PAN to preserve the membrane preparation possibilities, which reduces the potential amount of coupling groups (a). Direct heterogeneous esterification/amide formation and further conversion causes also PAN degradation and damages the membrane pore structure (b). AG bound via the acyl azide method to chemically modified PAN is not much influenced in its catalytic properties as expressed in the Michaelis–Menten constants.

Under UF conditions, AG effective activity can be improved due to the convective flow through the membrane. UF membrane selectivity for the macromolecular substrate starch determines the effective concentrations in the membrane, thus affecting the observed activities and product purities in the filtrate. Further studies with systematic variations of membrane pore structure, enzyme loading and activity, substrate size, and transport rates are underway and will enable us to optimize enzyme UF membrane performance.

LIST OF SYMBOLS

c	concentration
c_o	initial concentration
c_{ret}	concentration in the UF retentat
c_{filtr}	concentration in the UF filtrate
c_s	substrate concentration
d	diameter
$\varepsilon_{280\,nm}$	UV absorbance coefficient at 280 nm
φ	membrane selectivity for test substance: $\varphi = (c_{ret} - c_{filtr})/c_{ret} * 100\%$
J_v	filtrate flux
J_w	water flux
ν	valence bond (in IR spectra)
r	correlation coefficient
U	unit: 1 U = 1 μmol/min product formation by enzyme catalysis
v	reaction rate
v_{max}	maximum reaction rate

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